BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1986 by The American Society of Biological Chemists, Inc.

Vol. 261, No. 3, Issue of January 25, pp. 1177-1182, 1986
Printed in U.S.A.

The Primary Structure and Functional Characterization of the Neutral Histidine-rich Polypeptide from Human Parotid Secretion*

(Received for publication, May 24, 1985)

Frank G. Oppenheim‡§, Yueh-Chao Yang‡, Richard D. Diamond¶, David Hyslop¶, Gwynneth D. Offner§, and Robert F. Troxler§

From the ‡Department of Oral Biology, Goldman School of Graduate Dentistry, the ¶Department of Medicine and §Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118

The neutral histidine-rich polypeptide (HRP) from human parotid secretion was isolated by ion-exchange and gel-filtration chromatography. The complete amino acid sequence determined by automated Edman degradation of the protein, tryptic and *Staphylococcus aureus* V8 protease peptides, and digestion with carboxypeptidase A is:

1 5 10
NH_2-Asp-Pse-His-Glu-Lys-Arg-His-His-Gly-Tyr-Arg-Arg-
15 20 25
Lys-Phe-His-Glu-Lys-His-His-Ser-His-Arg-Glu-Phe-Pro-
30 35
Phe-Tyr-Gly-Asp-Tyr-Gly-Ser-Asn-Tyr-Leu-Tyr-Asp-Asn-COOH.

where Pse represents phosphoserine.

The polypeptide contains 38 residues and has M_r 4929. The charged amino acids predominate with 7 histidine, 4 arginine, 3 lysine, 3 aspartic acid, 3 glutamic acid residues, and 1 phosphoserine. Assuming minimal charge contributions from histidine and one negative charge from phosphoserine at pH 7, the net charge of HRP is balanced by an equal contribution of basic and acidic residues. Furthermore, the distribution of hydrophilic and hydrophobic residues along the polypeptide chain indicates that there is no structural polarity. The polypeptide lacks threonine, alanine, valine, cysteine, methionine, and isoleucine. HRP did not display sequence similarity with any protein sequence in the National Biomedical Research Foundation Data Bank.

HRP is an active inhibitor of hydroxyapatite crystal growth from solutions supersaturated with respect to calcium phosphate salts and therefore must play a role in the stabilization of mineral-solute interactions in oral fluid. In addition, HRP is a potent inhibitor of *Candida albicans* germination and therefore may be a significant component of the antimicrobial host defense system in the oral cavity.

The acquired enamel pellicle is a proteinaceous structure on tooth surfaces between the outer enamel surface and inner microbial layer, which is thought to control the mineral solution dynamics of enamel and exert selectivity on initial bacterial colonization (1). Human parotid saliva contains a group of anionic proteins which exhibit an unexpectedly high affinity for hydroxyapatite surfaces and which are implicated in the formation of the acquired enamel pellicle (2–5). The principal proteins which demonstrate this selective adsorption to hydroxyapatite surfaces are the four major anionic proline-rich proteins (PRPs[1]), the proline- and tyrosine-rich polypeptide statherin, and the neutral histidine-rich polypeptide (HRP) (6). The primary structures of the major anionic PRPs and statherin have been determined (7–10). These proteins are major salivary constituents (11–13), each contains two phosphoserine residues, and all are acidic with pI values ranging from 4.09 to 4.71 (11, 12). An unusual feature of the primary structures of these salivary components is the fact that most of their negatively charged amino acid residues, such as aspartic acid, glutamic acid, and phosphoserine, are contained almost exclusively within the amino-terminal region.

In addition to their role as pellicle precursors, these acidic salivary proteins and peptides are inhibitors of calcium phosphate precipitation from solutions supersaturated with respect to hydroxyapatite (2). The functional characteristics of the major anionic PRPs and statherin are incompletely understood at the molecular level. It is known, however, that the strongly negatively charged amino-terminal segment, particularly the phosphate groups, is important for this process (2).

HRP is similar to the PRPs and statherin by virtue of its high affinity for hydroxyapatite, but differs markedly in amino acid composition in that it contains only 1 residue of proline, has a pI of 7.0, and is the smallest known pellicle precursor protein (13). In addition to the neutral HRP, human parotid secretion also contains a group of basic histidine-rich proteins (with pI values greater than 9.5) which were at one time considered to be similar to histones (14) but were later shown to be intrinsic salivary components (15). Studies have shown that these basic HRPs exhibit a genetic polymorphism (16) and inhibit the growth of *Candida albicans* (17). The structural and functional relationship between the neutral and basic histidine-rich polypeptides has not been established.

The present investigation describes the isolation, characterization, amino acid sequence, and functional properties of the neutral HRP from human parotid saliva.

* This work was supported by National Institutes of Health Grants DE 05672, HL 13262, GM 22822, and AI 15338 and National Science Foundation Grant PCM83 14386. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: PRP, proline-rich protein(s); HRP, histidine-rich polypeptide; PTH, phenylthiohydantoin.

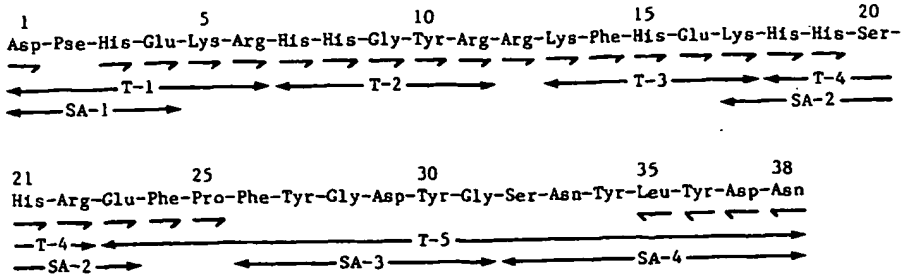

FIG. 1. **Amino acid sequence of HRP.** Designations are: *T*, tryptic peptides; *SA*, *S. aureus* V8 protease peptides; —, automated Edman degradation of intact HRP; ⟵, sequence obtained by carboxypeptidase A digestion.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

DISCUSSION

Of the major salivary components strongly implicated in the formation of the acquired enamel pellicle, HRP is the last to be fully characterized. This is due in part to difficulties encountered in isolating this polypeptide (13) which are attributable to several unique properties so far not observed with other salivary proteins and peptides. Specifically, HRP displays a consistent streaking pattern on disc gel electrophoretograms, displays an increasing resistance to solubilization from the lyophilized state at increasing degrees of purity, and displays anomalous chromatographic behavior on ion-exchange resins.

It seems likely that HRP aggregation gives rise to the streaking pattern on disc gels and that the solubility of HRP is facilitated by the presence of other proteins in salivary secretions. The aberrant chromatographic behavior of HRP is illustrated by the fact that this polypeptide essentially coelutes with statherin from DEAE-Sephadex A-25, but is almost completely resolved from statherin on Tris-Acryl M-DEAE (see Figs. 2 and 3 and "Results"). Since the functional group of these two anion exchangers is the same, the observed differences in affinity of HRP must be related to different interactions of the polypeptide with the two matrices. In addition, it should be noted that the pI of HRP is 7.0 (13) and the pI of statherin is 4.2 (12). Polypeptides of similar size differing in pI by 2.8 pH units should be well resolved from each other by ion-exchange chromatography, but such separation was not observed on either anion- or cation-exchange supports (see Figs. 2 and 3 and "Results").

The primary structure of HRP was established by automated Edman degradation of the polypeptide, tryptic and *S. aureus* V8 protease peptides, and by digestion with carboxypeptidase A (Fig. 1). These data provide the first complete amino acid sequence of a histidine-rich polypeptide in human salivary secretions. Several problems were encountered in determining this amino acid sequence. The first two attempts to sequence intact HRP resulted in large carry-overs beginning at cycle 1 (results not given). We have previously observed large carry-overs at cycles following phosphoserine residues (30) and found that this could be eliminated by performing double coupling and double cleavage at phosphoserine residues (30). Again, this problem was corrected by double coupling and double cleaving at the first two cycles of HRP (phosphoserine occurs at residue 2). This procedure may have broader application in the realm of sequence analysis of other phosphoproteins.

A search for sequence homology between HRP and protein sequences in the National Biomedical Research Foundation Data Bank using the IFIND program showed that the sequence of HRP is not related to the sequence of any known protein. Analysis of the primary structure by the Kyte and Doolittle (27) method indicated a minimal degree of hydropathy and predicted that the entire polypeptide chain is hydrophilic. Chou-Fasman analysis of HRP predicted two short segments of α-helix (residues 2–7 and 12–19), two short segments of β-pleated sheet (residues 26–29 and 35–38), and three reverse turns (residues 8–11, 20–23, and 31–34). These data suggest a high degree of ordered structure in HRP.

HRP is a potent inhibitor of crystal growth (see Table IV and "Results") which is consistent with its high affinity for hydroxyapatite surfaces (2). Both PRPs and statherin contain 2 phosphoserine residues, and enzymatic removal of the 2 vicinal phosphate moieties (residues 2 and 3) from the highly active amino-terminal tryptic hexapeptide of statherin and the 2 phosphates (residues 8 and 22) from the 30-residue amino-terminal tryptic peptides of the PRPs reduced inhibitory activity 60- and 100-fold, respectively (2, 5). HRP is unique in that it is the only known inhibitor of crystal growth which contains 1 phosphoserine residue. The availability of this protein will provide an additional opportunity to examine the molecular mechanism of crystal growth inhibition.

HRP was found to inhibit the germination of *C. albicans in vitro* (see Table V and "Results"). It is well known that certain cationic proteins and peptides display microbicidal activity. These include lysozyme (31, 32), permeability factors (33), chymotrypsin-like protein (34), and lysosomal cationic proteins (35). More recently, the cationic peptides MCP-1 and MCP-2 from rabbit lung macrophages (36) and NP-1, NP-2, NP-3a, NP-3b, NP-4, and NP-5 from rabbit peritoneal neutrophils (37) have been shown to kill *C. albicans in vitro*. The primary structure of these cationic peptides has been determined, and all are comprised of 32–34 amino acid residues, enriched with respect to cysteine and arginine, and display a high degree of sequence homology (38, 39). The primary structure of HRP and the cationic peptides is quite different. In HRP, cysteine is absent, the predominant amino acid is histidine rather than arginine, and 1 phosphoserine occurs. On the other hand, HRP is similar in size to the cationic peptides and also exhibits anti-Candida activity. The biological activity of HRP differs from that of the aforementioned peptides in that it inhibits *C. albicans* germination but does not kill in this assay. This is significant because germination optimizes adherance of *C. albicans* to oral mucous membranes (40, 41). Consequently, inhibition of germination would prevent colonization in the oral cavity. This is consistent with the observation that *C. albicans* can be cultured from oral

[2] Portions of this paper (including "Experimental Procedures," "Results," Tables I–V, and Figs. 2–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-1706, cite the authors, and include a check or money order for $6.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

fluid of most individuals, while candidosis in healthy individuals is rare.

The HRP characterized in this study is not the only histidine-rich polypeptide in human parotid secretion with antifungal activity. A family of closely related cationic HRPs with pI values greater than 9.5 (42) have been described (43, 44). The individual components have not been isolated and purified to homogeneity and, therefore, could be only partially characterized. The amino acid composition of this group of basic HRPs (43, 44) resembles that of the neutral HRP, and the unresolved mixture of basic HRPs exhibits fungistatic and fungicidal activity *in vitro* (17). Partial sequence analyses have been performed on partially resolved mixtures of basic HRPs, and although the results are difficult to interpret (42), they indicate that there is some sequence similarity with the neutral HRP. Earlier work indicated that the basic HRPs display a genetic polymorphism (16), as was found for the human anionic PRPs (45). The precise relationship between the neutral and basic HRPs has not yet been established. However, it is possible that a structural relationship exists similar to that identified for anionic (8, 9) and cationic proline-rich proteins (46, 47).

Acknowledgments—We wish to thank Dr. D. I. Hay, Forsyth Dental Center, for performing the inhibition of calcium phosphate precipitation and crystal growth assays on HRP, George Crombie for performing amino acid analyses, and Frederica MacMillan for expert technical assistance.

REFERENCES

1. Hay, D. I. (1983) in *Handbook of Experimental Aspects of Oral Biochemistry* (Lazzari, E. P., ed) pp. 319–335, CRC Press, Boca Raton, FL
2. Hay, D. I., Moreno, E. C., and Schlesinger, D. H. (1979) *Inorg. Perspect. Biol. Med.* **2**, 271–285
3. Hay, D. I., Schluckebier, S. K., and Moreno, E. C. (1982) *Calcif. Tissue Int.* **34**, 531–538
4. Moreno, E. C., Kresak, M., and Hay, D. I. (1984) *Calcif. Tissue Int.* **35**, 48–59
5. Aoba, T., Moreno, E. C., and Hay, D. I. (1984) *Calcif. Tissue Int.* **36**, 651–658
6. Hay, D. I. (1973) *Arch. Oral Biol.* **18**, 1517–1529
7. Schlesinger, D. H., and Hay, D. I. (1979) in *Peptides: Proceedings of the American Peptide Symposium, Washington, D. C.* (Gross, E., and Meienhofer, J., eds) Pierce Chemical Co., Rockford, IL
8. Wong, R. S. C., Hoffman, T., and Bennick, A. (1979) *J. Biol. Chem.* **254**, 4800–4808
9. Wong, R. S. C., and Bennick, A. (1980) *J. Biol. Chem.* **255**, 5943–5948
10. Schlesinger, D. H., and Hay, D. I. (1977) *J. Biol. Chem.* **252**, 1689–1695
11. Oppenheim, F. G., Hay, D. I., and Franzblau, C. (1971) *Biochemistry* **10**, 4233–4238
12. Hay, D. I. (1973) *Arch. Oral Biol.* **18**, 1531–1541
13. Hay, D. I. (1975) *Arch. Oral Biol.* **20**, 553–558
14. Balekjian, A. Y., and Longton, R. W. (1973) *Biochem. Biophys. Res. Commun.* **50**, 676–682
15. Baum, B. J., Bird, J. L., and Longton, R. W. (1977) *Arch. Oral Biol.* **22**, 455–456
16. Azen, E. A. (1972) *Science* **176**, 673–674
17. Pollock, J. J., Denepitiya, L., MacKay, B. J., and Iacono, V. J. (1984) *Infect. Immun.* **44**, 702–707
18. Oppenheim, F. G., Offner, G. D., and Troxler, R. F. (1982) *J. Biol. Chem.* **257**, 9271–9282
19. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404–427
20. Segrest, J. P., and Jackson, R. L. (1972) *Methods Enzymol.* **28**, 54–63
21. Allen, G. (1981) *Sequencing of Proteins and Peptides*, Elsevier/North-Holland, New York
22. Svanborg, A., and Svennerholm, L. (1961) *Acta Med. Scand.* **169**, 43–49
23. Perlman, G. E. (1952) *J. Am. Chem. Soc.* **74**, 6308–6312
24. Bhown, A. S., Mole, J. E., Weissinger, A., and Bennett, J. C. (1978) *J. Chromatogr.* **148**, 532–535
25. Chou, P. Y., and Fasman, G. E. (1979) *Adv. Relat. Areas Mol. Biol. Enzymol.* **47**, 45–148
26. Chou, P. Y., and Fasman, G. E. (1979) *Biophys. J.* **26**, 367–383
27. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132
28. Smith, D. L., and Rommel, F. (1977) *J. Immunol. Methods* **17**, 241–247
29. Lehrer, R. I. (1970) *Infect. Immun.* **2**, 42–47
30. Oppenheim, F. G., Offner, G. D., and Troxler, R. F. (1985) *J. Biol. Chem.* **260**, 10671–10679
31. Selsted, M. E., and Martinez, R. J. (1978) *Infect. Immun.* **20**, 782–791
32. Carroll, S. F., and Martinez, R. J. (1979) *Infect. Immun.* **24**, 460–467
33. Weiss, J., Elsbach, P., Olsson, I., and Odeberg, H. (1978) *J. Biol. Chem.* **253**, 2664–2672
34. Drazin, R. E., and Lehrer, R. I. (1977) *Infect. Immun.* **17**, 382–388
35. Odeberg, H., and Olsson, I. (1975) *J. Clin. Invest.* **56**, 1118–1124
36. Patterson-Delafield, J., Szlarek, D., Martinez, R. J., and Lehrer, R. I. (1981) *Infect. Immun.* **31**, 723–731
37. Selsted, M. E., Szklarek, D., and Lehrer, R. I. (1984) *Infect. Immun.* **45**, 150–154
38. Selsted, M. E., Brown, D. M., DeLange, R. J., and Lehrer, R. I. (1983) *J. Biol. Chem.* **258**, 14485–14489
39. Selsted, M. E., Brown, D. M., DeLange, R. J., Harwig, S. S. L., and Lehrer, R. I. (1985) *J. Biol. Chem.* **260**, 4579–4584
40. Kimura, L. H., and Pearsall, N. N. (1978) *Infect. Immun.* **21**, 64–68
41. Sobel, J. D., Meyers, P. G., Kaye, D., and Lesion, M. E. (1981) *J. Infect. Dis.* **143**, 76–83
42. Peters, E. H., and Azen, E. A. (1977) *Biochem. Genet.* **15**, 925–946
43. Baum, B. J., Bird, J. L., Millar, D. B., and Longton, R. W. (1976) *Arch. Biochem. Biophys.* **177**, 427–436
44. MacKay, B. J., Pollock, J. J., Iacono, V. J., and Baum, B. J. (1984) *Infect. Immun.* **44**, 688–694
45. Azen, E. A., and Oppenheim, F. G. (1973) *Science* **180**, 1067–1069
46. Isemura, S., Saitoh, E., and Sanada, K. (1980) *J. Biochem.* **87**, 1071–1077
47. Kauffman, D., Wong, R., Bennick, A., and Keller, P. (1982) *Biochemistry* **24**, 6588–6592

Continued on next page.

SUPPLEMENTARY MATERIAL TO:

The Primary Structure and Functional Characterization of the Neutral Histidine-Rich Polypeptide from Human Parotid Secretion.

By: Frank G. Oppenheim, Yueh-Chao Yang, Richard D. Diamond, David Bysiop, Gwynneth D. Offner and Robert F. Troxler

EXPERIMENTAL PROCEDURES

Materials

Carboxypeptidase A was purchased from Sigma. Escherichia coli alkaline phosphatase and L-1-tosyl-amido-2-phenylethyl chloromethyl ketone-trypsin were obtained from Worthington, and S. aureus V8 protease was obtained from Miles Laboratories. Human serum albumin was purchased from Calbiochem and lysozyme was from Boehringer-Mannheim. DEAE-Sephadex A-25 was from Pharmacia and Bio-Gel P-2, P-4, P-6, and CM-Bio Gel A were from BioRad. TRISACRYL M DEAE was purchased from LKB. Sequenator chemicals were purchased from Beckman Instruments and Burdick and Jackson. Parotid saliva was collected with the aid of a Carlson-Crittenden device as described previously (18). α_1-acid glycoprotein was a gift of Dr. K. Schmid, Boston University School of Medicine.

Isolaton of HRP

Method 1. Pooled human parotid saliva was dialyzed and lyophilized. Three grams of this material was fractionated on DEAE-Sephadex A-25 equilibrated with 0.05 M Tris-HCl, 0.025 M NaCl, pH 8.0, and was developed with a linear NaCl gradient (0.025-1.5 M) in the same buffer. Fractions enriched in HRP were pooled, desalted on Bio-Gel P-2, and chromatographed on CM Bio-Gel A equilibrated with 0.025 M sodium acetate, 0.025 M NaCl, pH 5.0, and developed with a linear salt gradient (0.025-0.8 M) in the same buffer. Final purification of HRP was achieved by gel filtration on Bio-Gel P-6 (200-400 mesh) equilibrated in and developed with 0.05 M ammonium bicarbonate, pH 8.0.

Method 2. Three grams of human parotid saliva protein was chromatographed on TRISACRYL M DEAE equilibrated in 0.05 M Tris-HCl, 0.025 M NaCl, pH 8.0, and developed with a linear NaCl gradient (0.025-0.75 M) in the same buffer. Fractions enriched in HRP were pooled, desalted on Bio-Gel P-2, and chromatographed on Bio-Gel P-6 (200-400 mesh) equilibrated and developed with 0.05 M ammonium bicarbonate, pH 8.0.

Elution profiles of individual chromatograms were monitored by continuous absorbance measurements at 227 or 230 nm, aliquots of column fractions were examined by disc gel electrophoresis (see below), and continuous conductivity measurements were made when gradients were employed.

Amino Acid Analysis

Protein and peptide samples were hydrolyzed in 0.5 ml of 6 N HCl at 110°C for 24 hours in evacuated tubes. Amino acid analyses were performed on either a Beckman 119 CL or Beckman System 6300 amino acid analyzer using a one column system.

Gel Electrophoresis

Column fractions were examined electrophoretically as described (18) in 7.5 % polyacrylamide gels including use of a stacking gel according to Davis (19). Gels were stained with 0.5 % Amido Black in 7 % acetic acid or with the periodic acid-Schiff reagent (20).

Phosphate Determination

Unbound (free) phosphate in HRP and derived peptides was measured by the method of Allen (21) by reference to a standard curve. Covalently bound phosphate was determined as described by Svanberg and Swennerholm (22) with the phosphoprotein standards, pepsin and PRP 1, which contain one and two mol of bound phosphate/mol, respectively (8, 23).

Carboxypeptidase A

Carboxypeptidase A digestion of HRP (130 nmol) was performed in 0.02 M N-ethylmorpholine acetate buffer, pH 8.5, at an enzyme to substrate ratio of 1:100. The 1.0 ml reaction mixture was incubated at room temperature, 0.2 ml aliquots were removed after 0, 5, 30, and 60 min, boiled, lyophilized, and the residues dissolved in 0.01 N HCl and subjected to amino acid analysis.

E. coli Alkaline Phosphatase

HRP (80 nmol) was dissolved in 3.0 ml of 1.0 M Tris-HCl, pH 8.0, and incubated with 2.8 units of enzyme for 60 min at 25°C on a rotating platform. The reaction mixture was applied directly to Bio-Gel P-2 and the product subjected to automated Edman degradation.

Trypsin

HRP (300 nmol) was dissolved in 1.0 ml of 0.05 M ammonium bicarbonate, pH 8.0, and digested with trypsin at an enzyme to substrate ratio of 1:50 for 4 h at 37°C. A second equivalent aliquot of enzyme was then added, and the reaction allowed to proceed for a further 4 h. The reaction was stopped by boiling, and the digest applied directly to a Bio-Gel P-4 column equilibrated and developed with 0.05 M ammonium bicarbonate.

S. aureus V8 Protease

HRP (170 nmol) was dissolved in 1.0 ml of 0.05 M ammonium bicarbonate and digested with S. aureus V8 protease at an enzyme to substrate ratio of 1:50 at 37° C for 18 h. The reaction was terminated by boiling and the digest applied directly to a Bio-Gel P-4 column equilibrated in and developed with 0.05 M ammonium bicarbonate.

Automated Edman Degradation

HRP and derived peptides were subjected to automated stepwise degradation on a Beckman 890C sequencer equipped with a cold trap using program No. 121078 as described previously (18). PTH-amino acids were identified by high pressure liquid chromatography (24).

Structural Evaluation

The amino acid sequence of HRP was analyzed for predicted secondary structure by the Chou-Fasman method (25, 26). The sequence was analyzed for hydropathicity by the method of Kyte and Doolittle (27) and was compared to the protein sequences contained in the National Biomedical Research Foundation data bank using programs obtained from Intelligenetics, Inc. (Palo Alto, CA).

Biological Activity

A. Inhibition of Calcium Phosphate Precipitation. The activity of HRP in the assay measuring inhibition of calcium phosphate precipitation from supersaturated solutions and in the assay measuring inhibition of hydroxyapatite crystal growth was determined as described by Hay (2).

B. Inhibition of Germination and Fungicidal Activity of HRP on Candida albicans. C. albicans (strain B-311) was maintained on Sabouraud's agar slants at 30° C and before use, cells were grown in the same medium without agar for 18-24 h at 30° C. The cells were harvested by centrifugation, washed three times with 0.15 M NaCl at 4°C, and suspended in 30 mM potassium phosphate buffer, pH 6.8, to a cell density of 10^5 cells/ml. Human serum albumin, egg white lysozyme, statherin, other salivary protein fractions or purified HRP were added to 1 ml cell suspensions at protein concentrations ranging from 0.002-5.0 mg/ml. After incubation for 1 h at room temperature, tubes were divided into two sets, one for the germination inhibition assay and one for the viability assay. In the germination assay, glucose (10 mg/100ml) and human serum (20% v/v) were added to initiate germination and the tubes were incubated for 0.5 to 4 h at 37°C. At intervals, aliquots were removed and the percentage of germinating blastospores were determined microscopically. In the viability assay, aliquots of cell suspensions previously incubated with test proteins were removed and the percentage of killed cells determined microscopically using the vital stains acridine orange (1.4 mg/dl) (28) and methylene blue (250 mg/dl) (29).

RESULTS

Isolation of HRP.

Method 1. The elution profile of human parotid saliva protein from DEAE-Sephadex A-25 reveals that HRP is a major component which elutes with statherin between 550-680 mEq NaCl (Fig. 2A). Partial separation of HRP and statherin was achieved by cation exchange chromatography on CM Bio-Gel A (Fig. 2B). Analysis of fractions by disc gel electrophoresis shows that statherin and HRP are not completely separated as evidenced by spreading and partial overlapping of both proteins over a region comprising 9-10 fractions. A minor component with an electrophoretic mobility intermediate between HRP and statherin was observed. Statherin and the minor component were adequately separated from HRP by gel filtration on Bio-Gel P-6 (Fig. 2C). The yield of HRP using Method 1 was approximately 1.5 mg/3 g of human parotid saliva protein.

Method 2. The elution profile of 3 g of human parotid saliva protein from TRISACRYL M DEAE (Fig. 3A) was, overall, similar to that from DEAE Sephadex A-25 (Fig. 2A). A major difference, however, is the complete separation of HRP from statherin on TRISACRYL M DEAE (Fig. 3A), which did not occur on DEAE Sephadex A-25 (Fig. 2A). HRP recovered from pooled fractions was separated from several additional components by gel filtration on Bio-Gel P-6 (Fig. 3B). The yield of purified HRP using Method 2 was approximately 9.8 mg/3 g of human parotid saliva protein.

While both Method 1 and 2 yielded highly purified HRP, the sequence determination and functional studies described below were carried out primarily with material derived by Method 1. Method 1 consistently provided HRP of a high degree of purity, whereas Method 2, which is only a 2 step procedure, did not always yield pure HRP. The data in Fig. 2 and 3 indicate that optimal and consistent isolation of HRP could be achieved by a 3 step chromatographic procedure using TRISACRYL M DEAE, CM Bio-Gel A, and Bio-Gel P-6.

Amino Acid Composition. Amino acid analysis of HRP showed that 55% of the polypeptide chain is comprised of the four amino acids, His (18%), Tyr (13%), Asx (13%) and Arg (11%), and lacks Thr, Ala, Val, Cys, Met, and Ile (Table I). The presence of only 3 residues of Phe and 1 residue of Leu indicate a minor contribution of hydrophobic amino acids. Minimum molecular weight computation based on 4.0 residues of arginine/mol resulted in a value of 4675 and a total of 37 amino acid residues.

Carbohydrate Analysis. It was concluded that HRP lacked carbohydrate because (a) no amino sugars were detected by amino acid analysis and (b) after disc gel electrophoresis (240 ug HRP/gel), no color reaction was observed with the periodic acid-Schiff stain under conditions where the control gel containing α_1-acid glycoprotein (70 ug/gel) gave an intense red-staining band.

Amino Acid Sequence. The amino acid sequence of HRP is given in Fig. 1 (Discussion). Sequential degradation of HRP established the sequence to residue 23 with two blanks at residue 2 and 20 (Table II). HRP was digested with trypsin and the resulting peptides were fractionated on Bio-Gel P-4 (Fig. 4A). Since the first eluting peak contained more than one component, the material in pooled fractions from this peak was recovered by lyophilization and chromatographed on Bio-Gel P-6 (Fig. 4B). The amino acid compositions and sequences of tryptic peptides are given in Table I and II, respectively. Peptides T-1 (residue 1-6), T-1a (residue 1-5), T-2 (residue 7-11), T-3 (residue 13-17) and T-4 (residue 18-22) could be aligned because they were contained within the amino-terminal sequence of the polypeptide (Fig. 1 - Discussion). Residue 2 and 20, not seen during sequential degradation of HRP were identified as follows: Residue 2 was tentatively identified as serine because a minute amount of PTH-serine was detected at cycle 2 of T-1. This residue was subsequently shown to be phosphoserine (see below). Residue 20 was positively identified as serine because PTH-serine was detected at cycle 3 of T-4. T-5 (residue 23-38) was identified as the carboxyl-terminal tryptic peptide because it lacked arginine and lysine. The sequence of T-5 provided a 3-residue overlap with the amino-terminal sequence of the protein and established the sequence of HRP to the carboxyl terminus.

HRP was digested with S. aureus V8 protease and the resulting peptides fractionated on Bio-Gel P-4 (Fig. 4C). Peptides SA-1 (residue 1-4), SA-2 (residue 17-23), SA-3 (residue 26-31) and SA-4 (residue 32-38) were recovered from pooled fractions and subjected to amino acid analysis (Table I) and automated Edman degradation (Table II). The S. aureus V8 protease peptide containing residues 5-16 was not found. Sequential degradation of SA-1 revealed that cycle 2 contained a minute quantity of PTH-serine (residue 2 of HRP). During sequential degradation of SA-2, PTH-serine was observed at cycle 4 (residue 20 of HRP), which confirmed identification of this residue in T-4. SA-3 resulted from the unexpected cleavage of a Pro-Phe (residue 25, 26) peptide bond and SA-4 resulted from spurious cleavage of a Gly-Ser (residue 31, 32) peptide bond. The sequences of SA-1, SA-2, SA-3 and SA-4 provided identification of certain residues seen previously in the amino-terminal sequence of HRP and in the sequences of tryptic peptides.

Two unexpected cleavages were observed when HRP was digested with S. aureus V8 protease. SA3 and SA4 arose from cleavage of Pro-Phe and Gly-Ser peptide bonds, respectively, whereas SA1 and SA2 arose from the predicted cleavage on the carboxyl side of glutamic acid residues. We have previously used this enzyme in protein sequence determinations but have never observed the non-specific cleavages mentioned above.

Carboxypeptidase A digestion of HRP provided the sequence, -Leu-Tyr-Asp-Asn-COOH, which confirmed the carboxyl terminal sequence seen previously in T-5 and SA-4.

Phosphate Determination. Phosphate analyses indicated that HRP contains one mol of bound phosphate per mol of protein (Table III). Phosphate analyses indicated T-1 (residue 1-6) contained 0.94 mol bound phosphate/mol, whereas the value obtained for T-4 (residue 18-22) was only 0.13 mol bound phosphate/mol. The latter value was considered insignificant because it was within the range of error of the method. For example experimental values for pepsin and PRP 1 were 1.08 and 2.13 mol bound phosphate/mol and these proteins contain 1 and 2 mol of bound phosphate, respectively. Collectively the results of phosphate analyses (Table III) and sequencing data (Table II) positively identified residue 2 of HRP as phosphoserine. It is possible serine at residue 20 (or residue 32) may have been dephosphorylated during isolation, although preferential removal of phosphate groups from residue 20 or residue 32, but not from residue 2, seems unlikely.

Biological Activity.

A. HRP was inactive in the assay which measures inhibition of the spontaneous precipitation of calcium phosphate salts from supersaturated solutions but active in the crystal growth assay (Table IV). In the latter assay, the HRP concentration at which 50 % inhibition was observed, was 1.8 uM. HRP is therefore more active than the PRPs for which the 50% inhibition values range from 2.5 to 11.8 uM (2) but considerably less active than statherin for which the 50 % inhibition value is 0.3 uM (2).

B. The effects of HRP in the assays measuring inhibition of blastospore germination and viability of C. albicans are shown in Table V. The data show that HRP is a potent inhibitor of germination since 50% and 80% inhibition was observed at the concentrations of 0.002 mg/ml (0.4 uM) and 0.008 mg/ml (1.6 uM), respectively. The standard test protein, lysozyme gave a 20% inhibition value at 1 mg/ml (83 uM). Thus, at the concentrations tested, HRP is 50-200 times more active than lysozyme. HRP was inactive in the viability assay under the conditions employed (Table V). It is noteworthy that statherin was inactive in both the germination and viability assays. Since cationic proteins and peptides of both salivary and non-salivary origin have been shown to exhibit microbicidal activity (see Discussion), the proteins not retained at pH 8.0 on DEAE Sephadex A-25 (fractions 8-27; Fig. 2A) were tested in both assays to probe for anti-Candida activity. Despite the fact that these non-retained fractions should contain all cationic salivary proteins, no significant activity could be detected even at high concentrations in either the germination inhibition or viability assay.

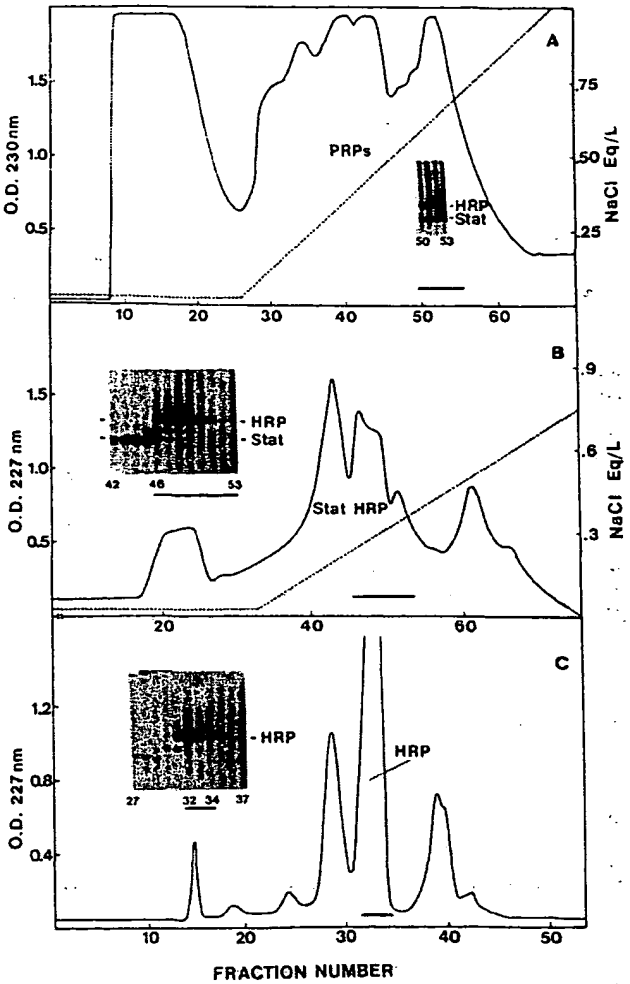

Figure 2. Isolation of HRP using Method 1. (A) Elution profile of parotid saliva protein from a DEAE Sephadex A-25 column (2.6 x 93.5 cm) equilibrated in 0.05 M Tris-HCl, 0.025 M NaCl, pH 8.0. The column was developed with a 48 h NaCl gradient in the same buffer using an LKB 11300 Ultrograd gradient mixer. The flow rate was 30 ml/h, 15 ml fractions were collected, and absorbance was monitored continuously at 230 nm with an LKB 2138 Uvicord S. In the chromatographic separations shown in Figure 2 and 3, 0.2 ml aliquots of selected fractions were analyzed electrophoretically (see Methods). In ion exchange separations, only the relevant portion of the salt gradient is shown (dashed line). The material contained in fractions 50-53 (bar) was desalted on Bio-Gel P-2 and chromatographed on CM Bio-Gel A. (B) Elution profile of partially purified HRP from a CM Bio-Gel A column (1.6 x 61.5 cm) equilibrated in 0.025 M sodium acetate, 0.025 M NaCl, pH 5.0. The column was developed with a 24 h NaCl gradient in the same buffer. The flow rate was 15 ml/h, 5 ml fractions were collected, and the eluate was monitored at 227 nm. (C) Final purification of HRP. Fractions 46-53 (bar) from (B) were subjected to gel filtration in 3 separate aliquots on a Bio-Gel P-6 column (1.6 x 94 cm) equilibrated in and developed with 0.05 M ammonium bicarbonate. The flow rate was 13.2 ml/h and 4.4 ml fractions were collected. HRP in fractions 32-34 was recovered by lyophilization.

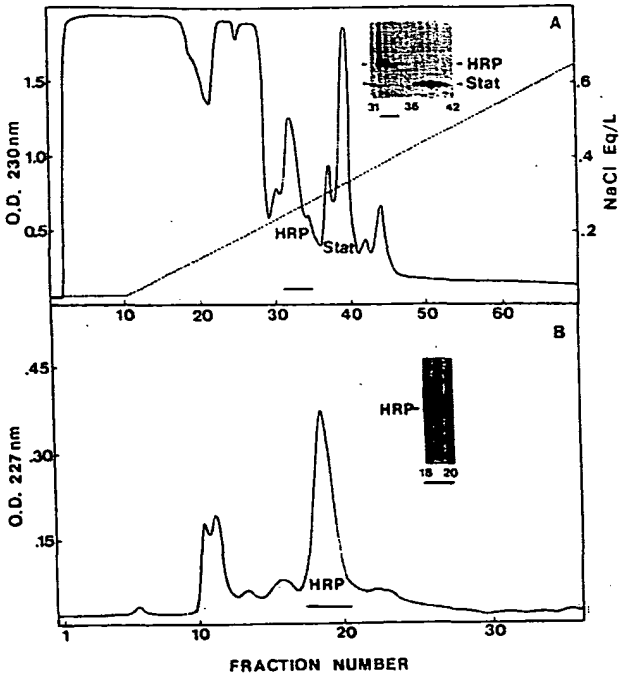

Figure 3. Isolation of HRP using Method 2. (A) Elution profile of parotid saliva protein from a TRISACRYL M DEAE column (2.6 x 94.0 cm) equilibrated in 0.05 M Tris-HCl, 0.025 M NaCl, pH 8.0 and developed with a 36 h NaCl gradient. The flow rate was 36.6 ml/h and 18.3 ml fractions were collected. Other chromatographic parameters were as described in Figure 2. (B) Final purification of HRP. Fractions 32-34 (bar) from (A) were subjected to gel filtration in 3 separate aliquots on a Bio-Gel P-6 column (1.6 x 88.5 cm) equilibrated and developed with 0.05 M ammonium bicarbonate. The flow rate was 10.5 ml/h and 3.5 ml fractions were collected. HRP in fractions 18-20 was recovered by lyophilization.

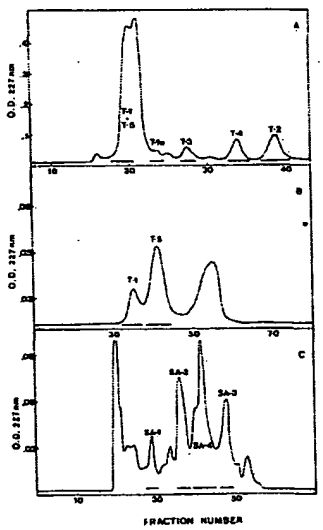

Figure 4. (A) Elution profile of tryptic peptides from a Bio-Gel P-4 column (1.5 x 94.5 cm) equilibrated and developed with 0.05 M ammonium bicarbonate. The flow rate was 12.0 ml/h and 4.0 ml fractions were collected. Peptides were recovered from pooled fractions indicated by bars. (B) Peptides T-1 and T-5 contained in the first eluting peak from (A) were separated on a Bio-Gel P-6 column (1.6 x 91.5 cm) equilibrated and developed with 0.05 M ammonium bicarbonate. The flow rate was 6.0 ml/h and 2.0 ml fractions were collected. (C)) Fractionation of *S. aureus* V8 protease peptides from a Bio-Gel P-4 column (1.6 x 93 cm) equilibrated and developed with 0.05 M ammonium bicarbonate. The flow rate was 10.0 ml/h and 3.3 ml fractions were collected. Peptides were recovered by lyophilization from pooled fractions as indicated by bars.

Table I. Amino acid composition of HRP, tryptic and *S. aureus* V8 protease peptides.[a]

Amino Acid	HRP (1-38)	T-1 (1-6)	T-2 (7-11)	T-3 (15-17)	T-4 (18-22)	T-5 (23-38)	SA-1 (1-4)	SA-2 (17-25)	SA-3 (26-31)	SA-4 (32-38)
Asx	5.3 (5)	1.0 (1)	0	0	0	4.0 (4)	1.2 (1)	0	1.0 (1)	2.7 (3)
Ser	2.9 (3)	0.9 (1)	0	0	1.0 (1)	1.1 (1)	0.8 (1)	1.3 (1)	0	1.3 (1)
Glx	3.1 (3)	1.0 (1)	0	1.2 (1)	0	1.9 (1)	1.3 (1)	1.4 (1)	0	0
Pro	1.2 (1)	0	0	0	0	1.6 (1)	0	0	0	0
Gly	2.8 (3)	0	1.1 (1)	0	0	2.3 (2)	0	0	2.4 (2)	0
Leu	1.1 (1)	0	0	0	0	1.1 (1)	0	0	0	1.0 (1)
Tyr	4.6 (5)	0	1.0 (1)	0	0	4.0 (4)	0	0	1.7 (2)	1.7 (2)
Phe	2.8 (3)	0	0	1.0 (1)	0	2.1 (2)	0	0	1.0 (1)	0
Lys	3.1 (3)	0.8 (1)	0	2.0 (2)	0	0	0	1.3 (1)	0	0
His	6.8 (7)	0.7 (1)	2.1 (2)	1.0 (1)	3.0 (3)	0	0.5 (1)	2.0 (3)	0	0
Arg	4.0 (4)	0.7 (1)	1.0 (1)	0	1.0 (1)	0	0	1.1 (1)	0	0

[a]Amino acid compositions were based on analysis of a single 20 h hydrolysate. Values in parentheses were deduced from the amino acid sequence.

Table II. Automated Edman degradation of HRP, tryptic and *S. aureus* V8 protease peptides.[a]

		1				5				10			
HRP	Amino Acid	Asp-	X -	His-	Glu-	Lys-	Arg-	His-	His-	Gly-	Tyr-	Arg-	Arg-	Lys-
	nmol	9.2		3.7	7.6	5.2	3.7	2.4	2.9	3.5	3.0	3.0	1.5	3.1
			15					20					25	
		Phe-	His-	Glu-	Lys-	His-	His-	X -	His-	Arg-	Glu-	Phe-	Pro-	
		3.1	2.4	1.8	2.5	2.3	2.7		1.6	0.8	1.0	0.8	2.0	
		1												
T-1	Amino Acid	Asp-	Ser-	His-										
	nmol	3.4	+	0.7										
		1				5								
T-2	Amino Acid	His-	His-	Gly-	Tyr-	Arg								
	nmol	4.0	3.1	2.5	2.2	0.6								
		1				5								
T-3	Amino Acid	Lys-	Phe-	His-	Glu-	Lys								
	nmol	12.1	5.9	9.3	5.4	2.5								
		1				5								
T-4	Amino Acid	His-	His-	Ser-	His-	Arg								
	nmol	4.1	3.8	0.5	1.3	0.4								
		1				5								
T-5	Amino Acid	Glu-	Phe-	Pro-	Phe-	Tyr-	Gly-	Asp-	Tyr-					
	nmol	11.9	7.4	3.8	6.2	6.6	2.4	4.4	5.0					
			10						15					
		Gly-	Ser-	Asn-	Tyr-	Leu-	Tyr-	Asp-	Asn					
		2.3	1.0	8.4	2.3	2.0	1.7	0.9	0.9					
		1												
SA-1	Amino Acid	Asp-	Ser-	His-	Glu									
	nmol	19.3	0.7	4.1	3.7									
		1				5								
SA-2	Amino Acid	Lys-	His-	His-	Ser-	His-	Arg-	Glu						
	nmol	57.6	22.2	25.1	1.8	10.7	8.9	6.3						
		1				5								
SA-3	Amino Acid	Phe-	Tyr-	Gly-	Asp-	Tyr-	Gly							
	nmol	21.3	15.3	6.1	8.7	11.8	5.1							
		1				5								
SA-4	Amino Acid	Ser-	Asn-	Tyr-	Leu-	Tyr-	Asp-	Asn						
	nmol	3.2	28.6	27.5	24.8	24.1	18.9	13.9						

[a] +, a PTH-derivative positively identified but not quantitated.

Table III. Phosphate Analysis of HRP.[a]

		Moles P/Mole of Protein	
Sample	Residue	Covalently Bound	Theoretical
HRP	1-38	0.93	-
T-1	1-6	0.94	-
T-4	18-22	0.13	-
Pepsin	-	1.08	1.0
PRP I	-	2.13	2.0

[a] Values represent the mean of 2 or more determinations.

Table IV. Inhibition of spontaneous precipitation and crystal growth in solutions supersaturated with respect to calcium phosphate salts by HRP, statherin and PRPs.

PROTEIN	SPONTANEOUS PRECIPITATION (uM)[a]	CRYSTAL GROWTH (uM)[a]
HRP	-	1.8
Statherin[b]	1.0	0.3
PRP 1-IV[b]	-	2.5-11.8

[a]Values indicate concentration for 50% inhibition.

[b]Data from reference 2.

Table V. Effect of HRP, statherin, salivary protein fractions not retained on DEAE Sephadex A-25 (SF1-SF4)[a] and lysozyme on the inhibition of blastospore germination and viability of *C. albicans*.

Protein	Concentration mg/ml	Inhibition of Germination % (S.E.)[b]	Cells killed % (S.E.)[b]
HRP	0.008	80.2 (5.8)	0
HRP	0.002	49.8 (2.6)	0
Statherin	0.15	5.2 (2.3)	0
SF1	5.0	9.8 (2.4)	2.5 (0.8)
SF2	5.0	14.4 (3.5)	0.5 (0.5)
SF3	1.0	9.6 (3.9)	1.4 (0.6)
SF4	5.0	0	0.5 (0.5)
Lysozyme	1.0	19.6 (6.6)	23.5 (1.3)

[a] SF1 to SF4 correspond to proteins from fractions 8-12, 13-17, 18-22 and 23-27 from the ion exchange separation shown in Figure 2A. These fractions contain only partially resolved salivary proteins. SF1 is enriched with amylase and lysozyme, and SF2 to SF4 contain salivary glycoproteins and uncharacterized basic proteins.

[b] Standard error of the mean of 4 determinations.